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Visual cycle retinoid processing proteins are present in HEK293S cells ☆

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Abstract

In HEK293S cells expressing opsin, rhodopsin regenerates on addition of all-*trans* retinol. This study was to determine if key proteins in the retinal pigment epithelium (RPE) are present in these cells. Cellular retinoid binding protein, cellular retinoic-acid binding protein, RPE65, caveolin-1-α- and -β-isoforms, interphotoreceptor retinoid binding protein, and 11-*cis* retinol dehydrogenase, but not lecithin:retinol acyltransferase (LRAT), were identified by Western blot analysis. LRAT transcripts were found by RT-PCR and Southern blot analysis. Small interference RNA specific to LRAT reduced ester formation, confirming that the enzyme is present. Therefore, HEK293S cells contain the functional components of the retinoid cycle found in the RPE.

Keywords: LRAT; HEK293; Retinol esterification; Visual cycle

1. Introduction

The exploration of retinoid metabolic mechanisms that occur in the retinal pigment epithelium (RPE) has been limited by the lack of cell systems containing the functional elements of the retinoid cycle pathway. For example, RPE65, which is both an abundant RPE protein (Hamel et al., 1993) and critical for the generation of 11-*cis* retinal (Redmond et al., 1998), is not found at the protein level in cultured RPE cells, although the gene has been detected (Hamel et al., 1993). The HEK293S cell line presented an attractive possibility based on two reports. First, Brueggemann and Sullivan (2001) had reported that in cells expressing opsin, rhodopsin could be formed from the addition of all-*trans* retinol, indicating that the retinol isomerization machinery is present in these cells. Secondly, we have previously reported the identification of RPE65 in these cells, both at the mRNA and protein levels (Ma et al., 1999). We therefore undertook to determine if some of the major proteins involved in the retinoid cycle in the RPE are present in HEK293S cells.

2. Methods

2.1. Cell culture and microsomal preparations

HEK293S cells (gift of Dr. Sullivan, Upstate Medical University, Syracuse, NY) were grown in a mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (1:1) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin, and incubated at 37 °C, 5% CO_2. The culturing of normal kidney, clear cell sarcoma of the kidney (CCSK) cells and congenital mesoblastic nephroma (CMN) cells were described previously (Hazen-Martin et al., 1994). The RNAs from the above kidney cell lines were from Dr. John Re, Medical University of South Carolina. HEK293S cells were scraped down into phosphate-buffered saline (PBS) solution and spun (600*g*, 10 min). Cell pellets were resuspended in Tris–HCl buffer (50 mmol/l, pH 7.4) containing 0.32 mol/l sucrose. Cells were subjected to freeze–thaw (three

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cycles) and spun (30 min, 30,000*g* at 4 °C). The supernatants were centrifuged at 100,000*g* for 60 min and the microsome pellets were washed twice with the above buffer, followed by centrifugation (100,000*g*, 2×, 15 min). Bovine and human RPE microsomes were prepared as described elsewhere (Moiseyev et al., 2003). The pellets were resuspended in buffer containing 50 mmol/l Tris-base, pH 8.0 with 1 mmol/l dithiothreitol (buffer A), sonicated on ice for 1 min, and the BioRad D_C Protein Assay was performed.

2.2. Western blot analysis

For the detection of retinoid metabolic proteins, 100 μg of total proteins from HEK293S cells, 5 μg bovine RPE microsomal proteins and 30 μg bovine RPE cyctosol were used for this study. For the detection of caveolin-1, 50 μg of HEK293S microsomal proteins and 15 μg bovine microsomal proteins were used for the reducing gel electrophoresis, followed by Western blot analyses. For the reducing and non-reducing gel electrophoresis and subsequent Western blots on lecithin:retinol acyltransferase (LRAT), 100 μg of microsomal proteins from HEK293S cells were used. The LRAT antibody was prepared by the MUSC antibody facilities using the same peptide as described previously (Ruiz et al., 1999). Dr. Bok (University of California at Los Angeles) generously provided antibody for comparison and no differences were found between the two antibodies. The antibodies for cellular retinol binding protein (CRBP) and cellular retinoic-acid binding protein (CRABP) were a gift from Dr. Saari, University of Washington, Seattle, WA. Antibodies to 11-*cis* retinol dehydrogenase (11-*cis* RDH) and RPE65 were gifts of Dr. Redmond, NEI. Antibody for interphotoreceptor retinoid binding protein (IRBP) was the gift of Dr. Wiggert, NEI. Antibody against caveolin-1 was purchased from BD Transduction Laboratories, San Diego, CA. For non-reducing gel electrophoresis, the above samples were mixed with SDS sample buffer without β-mercaptoethanol, resolved with 8–16% Novex Tris–glycine pre-cast gel, and were electrotransferred onto an Immobilon-P transfer membrane. For the strong denaturing, reducing gel electrophoresis, the above samples were mixed with SDS sample buffer containing tris(2-carboxyethyl)phosphine (TCEP, 120 mmol/l), resolved with 10% SDS-PAGE containing 6 mol/l urea in the stacking gel and electrotransferred onto the Immobilon-P transfer membrane. The membranes were blocked by incubation with 10% BLOTTO/TBST for 1 h and incubated with anti-LRAT polyclonal antibody (1:1000 dilution) overnight at 4 °C. Membranes were washed with TBST and incubated for 1 h in peroxidase-labeled anti-rabbit IgG secondary antibody (1:10,000 dilution). The membranes were washed and detected by the Renaissance Oxidizing Reagent and Enhanced Luminol Reagent (improved) developer substrates. SDS-PAGE and Western were performed in the presence of SDS and β-mercaptoethanol following the same procedure.

2.3. LRAT activity studies

[11,12-$^3H_2$]11-*cis* retinol was synthesized as described elsewhere (Bridges, Fong, & Alvarez, 1980). All the following procedures were performed under dim red light. [^{3}H] all-*trans* or 11-*cis* retinol (1 μCi, each) was applied to cultured HEK293S cells in one 100 mm dish directly, or to the total HEK293S proteins (50–12,000 μg) in 200 μl buffer A, and incubated for 5 h with the cells or 1 h with the cell lysate. Cells were washed with PBS and centrifuged at 600*g* for 5 min to get a cell pellet. Methanol (300 μl, 4 °C) was added to the cell pellet or to the 200 μl reaction mixture. The above mixtures were vortexed (1 min), hexanes (300 μl) were added, mixture vortexed (1 min), and sample centrifuged (5000*g*, 5 min). The upper phase was analyzed by high performance liquid chromatography (HPLC). Assessment of the effect of palmitoyl CoA was made by the addition 100 μmol/l palmitoyl CoA to each 200 μl of the microsomal fraction.

The extracted retinoids were separated on a 250 mm×4.6 mm Lichrosphere SI-60 5U normal phase column (Allen Asociates, Inc.) using hexane–ethyl acetate–dioxane–octanol: 85.4/11.2/2.0/1.4 as the mobile phase. For the collection of esters, methanol was substituted for octanol in the mobile phase. Retinoids were analyzed with a Waters HPLC system utilizing the Milennium software and a 996 Photodiode Array Detector monitoring at 320, 360 and 370 nm. An in-line radiomatic Packard 500TR Flow Scintillation Analyzer was used to monitor the [^{3}H] levels.

For the measurement of retinyl ester accumulation with time, 3×10^6 HEK293S cells were cultured for 24 h with 50 nmol/l [^{3}H] all-*trans* retinol. Cells were rinsed with PBS (3×) and incubated in the same growth media without [^{3}H] all-*trans* retinol for an additional four days. At various time points the media was removed, cells were harvested, and retinoids were extracted and identified.

For the calculation of the LRAT specific activity in HEK293S and bovine RPE microsomes, [^{3}H] all-*trans* retinol (1 μCi) in HEK293S (900 μg) or bovine RPE (5 μg) microsomes was brought to of 200 μl with buffer A and incubated with agitation (37 °C, 90 min). Reactions were stopped by addition of ice-cold methanol (300 μl). Retinoids were extracted with hexanes and analyzed by HPLC. By comparison to the standard [^{3}H] all-*trans* retinol (51816 cpm/pmol) and measuring the ester formed in the two samples, the LRAT specific activity in

HEK293S microsomes was calculated to be 1.849×10^{-3} pmol/mg/min, and the activity in bovine RPE microsomes to be 1.445 pmol/mg/min.

2.4. Ester saponification

Retinyl esters were collected from HPLC and dried under argon. The esters were mixed with 1 ml of 0.06 N ethanolic KOH and incubated at 55 °C for 1 h with agitation. Hexanes (5 ml) and water (1 ml) were added and centrifuged briefly. The upper organic phase was collected, dried under argon, redissolved in hexanes, and analyzed by HPLC.

2.5. LRAT inhibition studies

CRBP was expressed in *E. coli* M15 using the pQE30 CRBP expression plasmid (gift of Dr. Palczewski, University of Washington, Seattle, WA) and purified by Ni^{2+}-affinity chromatography (Crabb, Chen, Goldflam, West, & Kapron, 1998; McBee et al., 2000). 11-*N*-acetamido dodecylchloromethyl ketone (AcDCMK) was synthesized as described elsewhere (Moiseyev et al., 2003). *N*-ethylmaleimide (NEM) (1 μmol/l) and p-chloromercuribenzoate (pCMB; 10 μmol/l), AcDCMK (1.95 mmol/l), and apo-CRBP (19 μmol/l) were individually incubated with HEK293S cell total proteins (900 μg) in buffer A (200 μl, 30 min, 25 °C) and then with [^{3}H] all-*trans* retinol (1 μCi) in the dark (100 min, 37 °C) with agitation. The reactions were terminated with 300 μl of ice-cold methanol, and retinoids were extracted with 300 μl of hexanes for HPLC analysis.

2.6. RNA isolation, RT-PCR, Southern blotting and cDNA sequencing analysis for LRAT

Total RNAs from normal kidney, CCSK, CMN and HEK293S cells were isolated by the acid guanidinium isothiocyanate (GIT)-phenol/chloroform extraction method (Chomczynski & Sacchi, 1987). Total RNA (5 μg) from each cell line was used to perform reverse transcription (RT) as described previously (Ma et al., 1999). The PCR primers were designed based on the gene bank human LRAT sequence: 5′ primer (F1, in exon 1): AGGATGAAGAACCCCATGCTGGAG, and 3′ primer (T1, in exon 3): CCAGAACACAGTGTTACGGGTCAC. The mixture of 5 μl of the RT product, 10 pmol of each F1 and T1 primer, and 3 μl of 2.5 mmol/l dNTP were subjected to PCR in a final volume of 25 μl using the Expand High Fidelity PCR System. The PCR was carried out for 1 cycle at 95 °C, 5 min, followed by 36 cycles at 95 °C, 1 min; 61 °C, 1 min; 72 °C, 1 min; and 1 cycle at 72 °C for 3 min in a Robocycler. All PCR products were probed with ^{32}P-labelled nested primers in the Southern blot analysis. The nested primer T3 for Southern blot analysis was also designed according to gene bank human LRAT sequences, and the sequence of primer T3 is 5′-GAGCAGTGCCTTTTTCTG-3′. The above PCR products were purified with a PCR purification kit and sequenced with T3 (reverse) and T2 (forward) primers. The sequence of T2 primer is 5′-AACAAGCGTCT- CATCCTG-3′. The sequencing results were compared with the human LRAT gene bank sequence (gene bank access number AF071510) using GCG software.

2.7. LRAT RNA interference (RNAi) assay

Two sequences of LRAT gene specific 21-oligonucleotide small interference RNA (siRNA) with two thymidine residues (dTdT) at the 3′ end of the sequence were designed and synthesized by Eurogentec. The sequence of the sense strand is: 5′-ACCAGCUCUUUCCACCGAGdTdT-3′, and the sequence of the anti-sense strand is: 3′-dTdTUGGUCGAGAAAGGUGGCUC-5′. Each strand of siRNA was diluted to 50 μmol/l with RNase-free water and with annealing buffer supplied by the manufacturer to 20 mol/l. The final salt concentration was 50 mmol/l Tris, pH 8.0, 100 mmol/l NaCl. The above mixture was incubated (2 min, 95 °C) and cooled slowly to room temperature (60 min to 25 °C) in a Robocycler. After annealing, the siRNA duplex was stored on ice until use. Double-stranded control siRNA (sense: 5′-UUCUCCGAACGUGUCACGUdTdT-3′, anti- sense: 3′-dTdTAAGAGGCUUGCACAGUGCA-5′) that is not homologous to any of the known genes was purchased from Xeragon, Qiagen. The control siRNA duplex (50 nmol) was taken up in buffer (250 μl) containing 100 mmol/l potassium acetate, 30 mmol/l of HEPES-KOH, and 2 mmol/l magnesium acetate, pH 7.4, heated to 90 °C for 1 min and incubated at 37 °C for 60 min.

HEK293S cells (1×10^3) were plated onto a 100 mm dish and grown in culture medium containing DMEM/F12 (1:1), 10% FCS without antibiotics the day before transfection. Oligofectamine reagent (45.5 μl) was diluted into 179.5 μl Opti-MEM-I reduced serum medium, and incubated at room temperature for 10 min. The LRAT siRNA and the control RNA duplexes (75 μl, 20 μmol/l) were diluted into 1.2 ml of Opti-MEM-I medium, mixed with the above Oligofectamine, and incubated (20 min, 21 °C). After washing the cells with 5 ml of Opti-MEM-I, Opti-MEM-I medium (6 ml) and the above mixtures containing RNA and Oligofectamine (1.5 ml) were applied to each plate of cells with mixing. Cells were incubated (5% CO_2, 37 °C, 24 h), re-transfected with the above reagent, and incubated for 48 h. The cells were then assayed for LRAT activity as above.

3. Results

Western blot analyses were performed on the HEK293S cell lysates in order to detect retinoid cycle proteins at the protein level. The proteins studied were CRBP, CRABP, IRBP, 11-*cis* RDH and RPE65 and caveolin-1. The bovine RPE microsomal fraction was used as a positive control. CRBP, CRABP, IRBP, 11-*cis* RDH and RPE65 were identified in the HEK293S cells, indicating their potential involvement in retinoid metabolism in the HEK293S cells (Fig. 1). Caveolin was studied as its possible relationship with RPE65 has been suggested (Redmond, personal communication). The caveolin-1-α- and -β-isoforms were found to be present in both the RPE and HEK293S cells. It is difficult to compare the absolute levels of these retinoid cycle proteins, in the HEK293S cell lysates and the RPE microsomes. However, it should be noted that the ratio of cell lysate to RPE microsomes was 20:1, so the level of the proteins in the HEK293S cells is definitely lower than in the RPE.

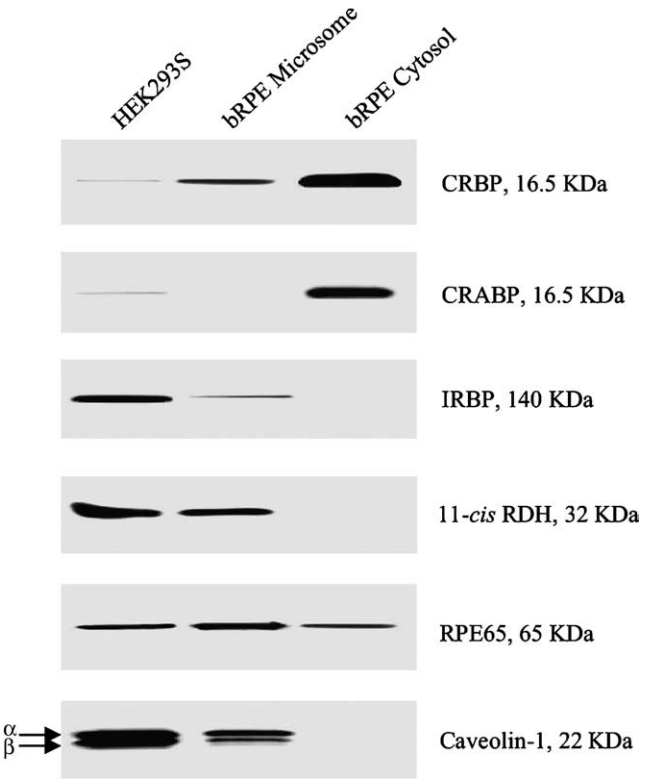

Fig. 1. Detection of retinoid metabolic proteins by Western blots. Total proteins from HEK293S cells (100 μg), bovine RPE microsomes (5 μg), and bovine RPE cytosol (30 μg) were resolved with 12% SDS-PAGE, transferred onto nitrocellulose membranes and probed with polyclonal antibodies that recognize CRBP, CRABP, IRBP, 11-*cis* RDH, RPE65, respectively (1:1000 dilution). For caveolin-1, 50 μg of HEK293S microsomes, 15 μg of bovine microsomes, and 30 μg of bovine RPE cytosol were used. The antibody against caveolin-1 recognizes both α- and β-isoforms of this protein.

Western blot analysis was also performed to detect LRAT protein in HEK293S cell lysate, as this is a key (Canada et al., 1990; Ruiz et al., 1999) and abundant enzyme in RPE. In the presence of SDS and without β-mercaptoethanol, the LRAT polyclonal antibody detected multiple bands from HEK293S cells as well as bovine RPE, and human RPE microsomes (Fig. 2A). In the presence of urea and TCEP (stronger denaturing reducing conditions), the LRAT antibody detected a single band in each sample. In the human and bovine RPE microsomes, the 25 KDa LRAT protein was detected; however, in HEK293S, only a 30 KDa protein band was observed (Fig. 2B). This same band had been previously shown by mass spectrometric sequencing to be a myosin heavy-chain fragment (Jahng et al., 2002), which apparently cross-reacts with this antibody.

Retinyl ester formation was formed with the cultured HEK293S cells on the addition of all-*trans* retinol (Fig. 3A(i)) or 11-*cis* retinol (Fig. 3A(iii)). Similar data were

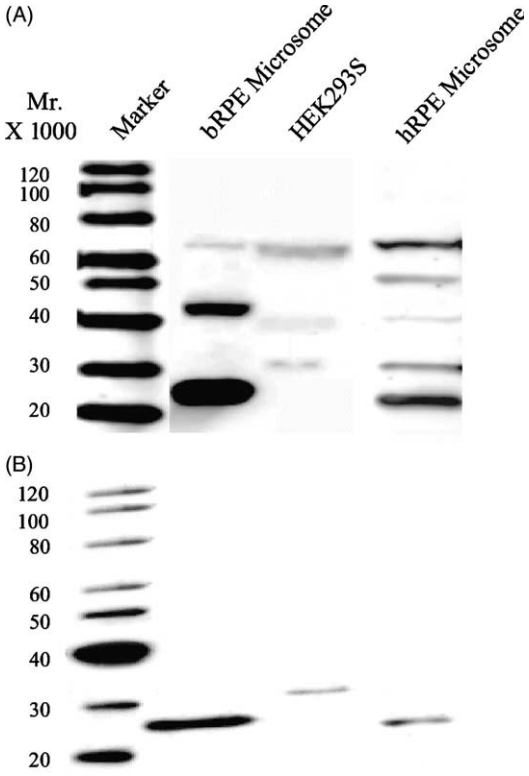

Fig. 2. LRAT in HEK293S cells is not detected on Western blots. Microsomal proteins from HEK293S (100 μg), bovine RPE (5 μg) and human RPE (5 μg) were used for Western blot analysis. (A) SDS denaturing and non-reducing conditions—samples were resolved with 8–16% Tris–glycine gel, and exposed to the LRAT antibody (1:1000 dilution). Multiple bands were detected in each sample. (B) Urea and TCEP reducing conditions—samples were resolved with 10% SDS-PAGE containing 6 mol/l urea and exposed to the LRAT antibody (1:1000 dilution). A single band was detected in each sample at 25 KDa in the human and bovine RPE microsomes and at 30 KDa in the HEK293S cells. The 30 KDa band has been shown to be the myosin heavy-chain (Jahng, Cheung, & Rando, 2002).

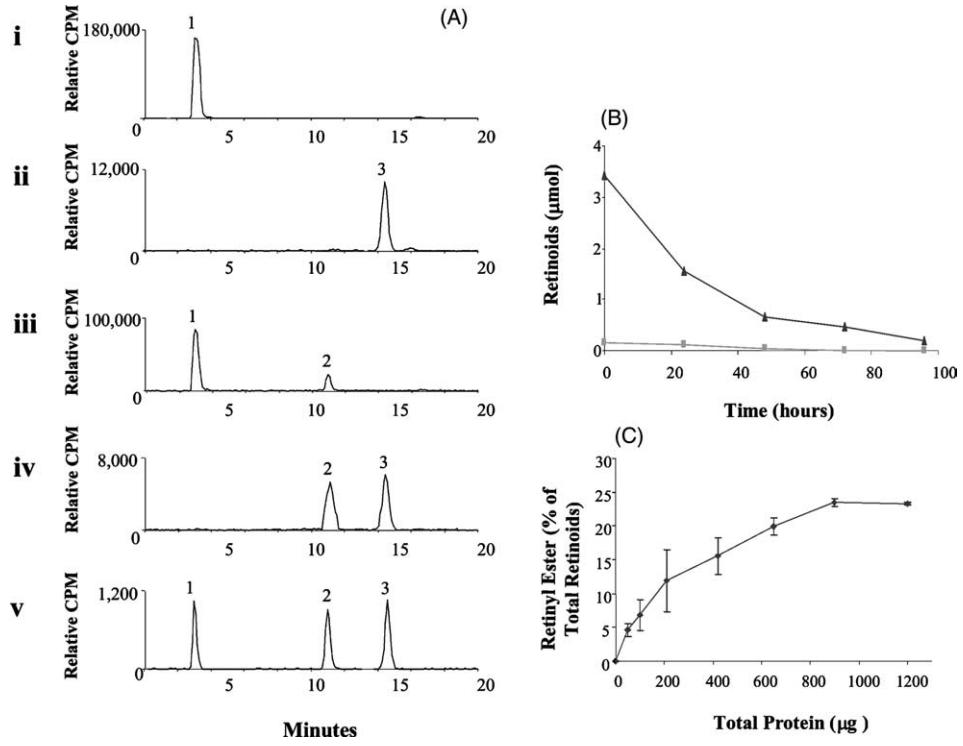

Fig. 3. [^{3}H] all-*trans* retinol metabolism in HEK293S cells. (A) All-*trans* and 11-*cis* retinol are substrates of LRAT. (i) [^{3}H] all-*trans* retinol was applied to the HEK293S cell culture and incubated for 5 h; retinoids were extracted and analyzed with HPLC. (ii) The [^{3}H]retinyl ester generated from (i) was collected and saponified. The saponified retinoids were extracted and analyzed with HPLC. (iii) [^{3}H]11-*cis* retinol was applied to the HEK29S cell culture and incubated for 5 h; retinoids were extracted and analyzed with HPLC. (iv) The [^{3}H]retinyl esters generated were collected and saponified. The saponified retinoids were extracted and analyzed with HPLC. (v) Standards: Peak 1, all-*trans* retinyl ester; Peak 2, 11-*cis* retinol; Peak 3, all-*trans* retinol. (B) Retinol metabolism in HEK293S cells. Cells were cultured for 24 h with all-*trans* retinol. The medium was removed and cells continued to grow in growth medium without additional all-*trans* retinol for 24, 48, 72 and 96 h respectively. The intracellular all-*trans* retinyl esters and all-*trans* retinol were plotted against time. The experiment was performed twice with similar results. –▲– retinyl esters, —■— all-*trans* retinol. (C) LRAT activity correlation with HEK293S protein concentration. Ester formation increases as HEK293S protein increases. Ester accumulation was plotted against increasing amounts of total HEK293S proteins ($n = 3$).

obtained when the labeled retinols were added to the HEK293S microsomes (data not shown).

In order to determine the isomeric integrity of the retinol in the newly formed esters, the retinyl esters were saponified and the products analyzed by HPLC. Results showed that all-*trans* retinyl esters were generated from all-*trans* retinol (Fig. 3A(i) and (ii)). The 11-*cis* retinyl esters were generated from 11-*cis* retinol (Fig. 3A(iii) and (iv)). However, all-*trans* retinol was produced as well which could have occurred in the incubation of the retinol with the cells or in the saponification process. As the incubations were conducted at 37 °C and the saponification at 55 °C, it is likely that some thermal-isomerization occurred.

To follow ester formation in the HEK293S cells, [^{3}H] all-*trans* retinol was added to the culture medium and incubated for 5 h. Unlabeled all-*trans* retinol was then added and the level of labeled retinoid in the ester form was followed over time. The intracellular [^{3}H] all-*trans* retinyl ester levels decreased significantly within 96 h, and the intracellular [^{3}H] all-*trans* retinol levels remained low and barely detectable after 96 h of incubation (Fig. 3B). Similar results were obtained with 11-*cis* retinol (data not shown). The all-*trans* retinyl esters accumulation increased with increasing levels of protein from the HEK293S cells (Fig. 3C), demonstrating that the enzyme(s) responsible for this reaction are present in those cells.

To prove the existence of LRAT activity in HEK293S cells, thiol reagents NEM and pCMB, LRAT specific synthetic inhibitors AcDCMK and apo-CRBP protein were pre-incubated with HEK293S cell lysate for 30 min at effective concentrations of 1, 10 μmol/l, 1.95 mmol/l and 19 μmol/l, respectively. HPLC analysis revealed decreased ester formation after incubating with the above inhibitors (Fig. 4). The sensitivity of LRAT activity to NEM and pCMB indicates the involvement of a sulfhydryl group in the site of catalysis. The specific inhibition by AcDCMK and apo-CRBP further indicates that LRAT contributes to the synthesis of retinyl esters from retinol.

Palmitoyl CoA is essential for ARAT activity (Ross, 1982). Therefore, to test whether ARAT exists in the HEK293S cells, palmitoyl CoA was incubated with

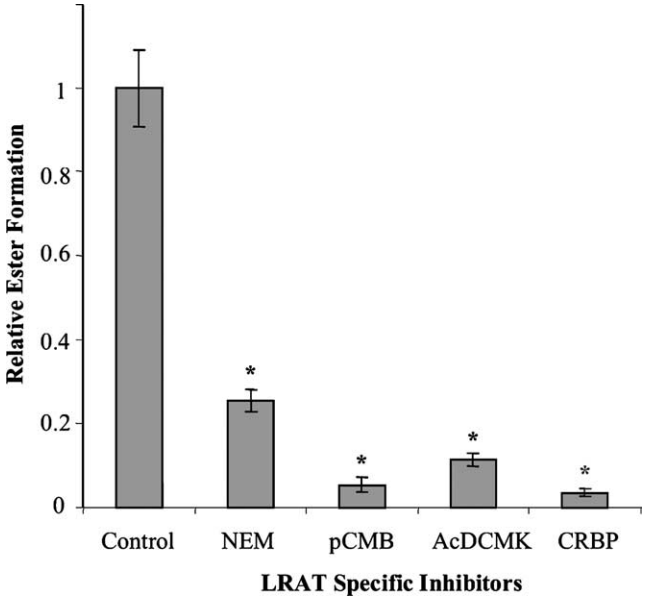

Fig. 4. Inhibition of retinyl ester formation by LRAT inhibitors. NEM (1 μmol/l), pCMB (10 μmol/l), AcDCMK (1.95 mmol/l) and apo-CRBP (19 μmol/l) were preincubated with HEK293S cell total proteins at room temperature for 30 min. [^{3}H] all-*trans* retinol was added to the reaction mixture and incubated for an additional 100 min at 37 °C. The levels of [^{3}H] all-*trans* retinyl esters were assessed by HPLC ($n = 3$). ∗ indicates $P < 0.01$.

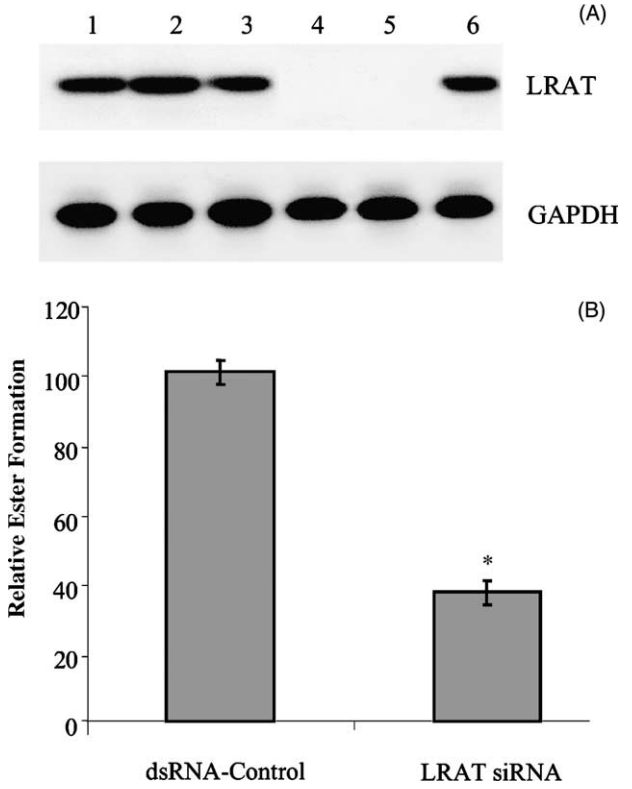

Fig. 5. LRAT RNA is detected and is inhibited by siRNA. (A) RT-PCR and Southern blot analysis of LRAT in normal human kidney, HEK293S and human primary tumor cells. The primers specific for LRAT and GAPDH amplified the cDNAs of LRAT, and GAPDH, from normal kidney (lane 1), HEK293S (lane 2), CCSK-BG1 (lane 3) and CCSK-2 (lane 6), but not from CMN-1 (lane 4) and CMN-2 (lane 5). (B) LRAT siRNA reduced retinyl ester formation in HEK293S cells. LRAT specific siRNA duplex and double-stranded RNA (dsRNA) control were transfected into HEK293S cells, incubated for 72 h, and assayed for in vitro LRAT activity. The relative retinyl esters formed in the reactions with LRAT siRNA compared with the dsRNA control were plotted ($n = 2$ with 3 measurements in each experiment). ∗ indicates $P < 0.05$.

HEK293S protein and [^{3}H] all-*trans* retinol. HPLC analyses of the retinoids extracted from the above reactions showed no significant increase in the formation of retinyl ester after inclusion of palmitoyl CoA in the reaction (data not shown). Thus, ARAT is not responsible for the generation of retinyl esters in the HEK293S cells.

RT-PCR using primers specific to the gene bank human LRAT sequence (GenBank accession number AF071510) amplified LRAT cDNA from normal kidney, CCSK cells, and transformed kidney cells, but not from the CMN cells. Southern blot analysis of the PCR products hybridized with a nested oligonucleotide probe specific for LRAT generated a single band, and confirmed the specificity of the PCR results (Fig. 5A). Southern analysis of GAPDH was performed simultaneously as a control. To further prove the above results were correct, the PCR products from normal kidney, CCSK and HEK293S cells were sequenced and aligned with the human LRAT gene bank sequence. The perfect sequence alignment of the PCR products with the human LRAT cDNA sequence from the start-to-stop codon confirmed that the PCR amplified the full-length LRAT cDNA of total RNA of HEK293S cells (data not shown).

To prove that the esterifying activity observed was indeed coming for the LRAT protein, a RNA interference experiment (Elbashir et al., 2001; Montgomery, Xu, & Fire, 1998) was performed, followed by an in vitro LRAT activity assay. LRAT specific siRNA duplex and double-stranded, control RNA were transfected into HEK293S cells, incubated for 72 h, and assayed for LRAT activity. HPLC analyses of the extracted retinoids revealed significantly decreased retinyl ester formation in the LRAT siRNA transfected HEK293S microsomes (Fig. 5B). The above results further confirmed the existence of specific LRAT activity in HEK293S cells.

4. Discussion

The rather surprising data of Sullivan and co-workers (Sullivan & Shukla, 1999) are quite convincing that retinoid metabolism can occur in the HEK293S cells. These workers observe that in HEK293S cells expressing opsin, bleached rhodopsin would spontaneously regenerate. These workers went on to demonstrate that in-

deed the addition of all-*trans* retinal and all-*trans* retinol led to the formation of rhodopsin and hence, presumably, 11-*cis* retinal (Brueggemann & Sullivan, 2001, 2002). The 11-*cis* form of retinal is unstable, and difficult to synthesize in vitro. Furthermore, 11-*cis* retinal is not known to be generated, or have a role, outside of the retina and RPE, except in the pineal gland (Okano & Fukada, 1997; Su, Chai, Kahn, & Napoli, 1998). We had been studying the expression of RPE65 and found that this protein is expressed in HEK293S cells. We therefore undertook to determine if other enzymes and binding proteins known to be involved in the generation of 11-*cis* retinal in the RPE, are likewise present in HEK293S cells.

Western blot analysis showed the presence at the protein level of CRBP, CRABP, IRBP, 11-*cis* RDH and RPE65 in HEK293S cells. The amounts present are greatly reduced as compared with the RPE cells, but their presence is certainly detectable. We also tested for cavoelin, as this protein has recently been suggested to have an association with RPE65 (Redmond, personal communication), and this protein was also found to be present.

Interestingly, we were unable to find a band at 25 KDa in the HEK293S cells using the Western blot approach with the LRAT antibody. This was perplexing, as it has now been shown by two groups using different methods that in the RPE, the ester is the substrate of the isomerase which converts all-*trans* retinyl ester into 11-*cis* retinol (Gollapalli & Rando, 2003; Moiseyev et al., 2003). LRAT activity was detected using several methods. Retinyl esters were formed from both 11-*cis* and all-*trans* retinol and this activity could be reduced with known inhibitors of LRAT. The failure of palmitoyl CoA to increase the esterfication reaction suggests that the enzyme is not ARAT, but is indeed LRAT. Our LRAT antibody detected a band at 30 KDa in HEK293S cells, but this band has previously been sequenced by mass spectrometry and found to be myosin heavy-chain (Jahng et al., 2002).

LRAT cDNA has been cloned from human RPE (Ruiz et al., 1999), bovine RPE (Ruiz et al., 2001), and rodent liver (Zolfaghari & Ross, 2000), and found to be ~2.5 kb. Recently, another 5.3 kb LRAT cDNA was cloned from rat liver (Zolfaghari, Wang, Chen, Sancher, & Ross, 2002) with a much longer 3′-untranslated region. However, both forms of cloned LRAT mRNA were proven to express the functional 25 KDa LRAT proteins (Ruiz et al., 1999; Zolfaghari et al., 2002). The existence of multiple forms of LRAT mRNA suggests the complexity of LRAT transcriptional regulation and gene expression in different tissues.

To confirm that LRAT is present in the HEK293S cells, primers F1 and T1 were designed according to human LRAT cDNA sequence and LRAT genomic DNA sequences at chromosome 4 (Ruiz et al., 2001). Several pairs of primers were also designed according to an EST sequence N78382, which contains an alternative spliced form of LRAT cDNA by comparing with LRAT genomic sequence. RT-PCR, gene sequencing and Southern analysis were performed using total RNA purified from HEK293S cells, but no alternative forms of LRAT cDNA were found. The full-length LRAT cDNA sequence, identical to the human Genbank sequence, was identified in HEK293S cells. Also, no cDNA identical to N78382 was amplified from either HEK293S cells or human RPE RNAs by RT-PCR and may be a rare alternative splicing form caused by mutation of the LRAT gene. Previous studies had failed to detect LRAT in these cells by RT-PCR and/or Northern blot analysis (Ruiz et al., 1999; Zolfaghari et al., 2002). The fact that LRAT gene transcripts were so difficult to detect indicates that LRAT exists at an extremely low level in these cells. Estimates of the specific activity confirmed that in bovine RPE, LRAT specific activity is 1.4 pmol/mg/min, about 1000 times higher than the activity of 1.85×10^{-3} pmol/mg/min found in the HEK293S microsomes.

We performed the same experiments on normal kidney and kidney tumor cells (CCSK and CMN). The LRAT cDNA identical to that of the 25 KDa human RPE LRAT was detected in the cells of normal kidney and the CCSKs, but not in the CMNs. These results are in agreement with the previous finding that LRAT activity is low in renal cancer cell lines as compared with normal kidney cells (Guo et al., 2001).

HEK293 cells have recently been described as possible neuronal progenitors of retinal lineage (Shaw, Morse, Ararat, & Graham, 2002). This finding indicated that HEK293 cells should not be referred to as a kidney cell line (Toth & Wold, 2002). The fact that these cells are likely to be of retinal origin explains the presence of these proteins that are key to the retinoid cycle in the RPE. In HEK293S cells, the endogenous levels of these proteins are low, particularly LRAT. However, this cell culture system has the retinol isomerase activity characteristic of the RPE as well as several key visual cycle proteins. Thus this cell system may provide a useful tool for studies of the visual cycle.

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